Down-Regulation of Tomato β-Galactosidase 4 Results in Decreased Fruit Softening[1]

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Transcript abundance of the gene encoding β-galactosidase II, a β-galactosidase/exo-galactanase (EC 3.2.1.23) present during tomato (*Lycopersicon esculentum*) fruit ripening, was suppressed by expression of an antisense tomato β-galactosidase 4 (TBG4) cDNA driven by the cauliflower mosaic virus 35S promoter. RNA gel-blot analysis was used to evaluate TBG4 mRNA levels in transgenic fruit. All of the antisense lines had attenuated TBG4 mRNA levels in turning stage fruit; however, TBG4 mRNA suppression was unstable, and mRNA levels varied in red-ripe fruit among the lines. Suppression of TBG4 mRNA levels in antisense fruit was correlated with a reduction in extractable exo-galactanase activity against a lupin galactan. All of the antisense lines had reduced free galactose levels at mature green stage 4, but levels comparable with controls during ripening. Total cell wall galactosyl contents in the antisense fruit were not significantly different from control fruit. Whole-fruit firmness was measured using a texture analyzer and the means of the peak force measurements for four of six antisense lines were significantly higher than control fruit. One antisense line had red-ripe fruit that were 40% firmer than controls. Fruit from this antisense line also had the lowest TBG4 mRNA and exo-galactanase levels and the highest wall galactosyl content during the early stages of ripening, implicating an involvement of this gene product in cell wall modification leading to fruit softening.

Fruit ripening includes changes that enhance visual appearance, flavor and textural changes to favor consumption. Textural changes are believed to be the result of changes in turgor pressure and cell wall and membrane composition and degradation. Although it is believed that the overall softening process results from a number of changes in all three factors, cell wall modifications have been implicated to be the major determinant of fruit softening. Although no single protein has been shown to be a major determinant of tomato (*Lycopersicon esculentum*) fruit softening, nonetheless, it has been demonstrated that changes in the concentration of a single class of proteins can have a significant effect on the final textural qualities of fruit.

Antisense suppression of polygalacturonase accumulation demonstrated that whereas the enzyme contributes little to fruit softening (Kramer et al., 1992; Langley et al., 1994), it substantially affects the textural properties (increased viscosity) of pastes, the integrity of stored fruit, and resistance to postharvest pathogens (Schuch et al., 1991; Kramer et al., 1992; Langley et al., 1994). Suppression of pectin methylesterase activity had little effect on fruit firmness or ripening characteristics, but did result in significant favorable changes in the soluble solids content of raw juice and serum viscosity, paste viscosity, and serum separation of processed juice (Tieman et al., 1992; Thakur et al., 1996). In addition, reduction in ripening-related expansin LeExp1 accumulation by transgenic suppression caused a significant difference in fruit softening throughout ripening (Brummell et al., 1999).

Tomato fruit development is marked by significant changes in the galactosyl content of the pericarp cell wall (Kim et al., 1991). From 10 d post-pollination (dpp) to the mature green stage (a period approximately 40 ± 5 d in most cultivars) the galactosyl content measured as a percentage of the entire wall is reduced 2-fold. Moreover, during the period between the onset of ripening, i.e. breaker stage, and the mature red-ripe stage (approximately 8 ± 2 d), the wall galactosyl content is again reduced 2-fold (Kim et al., 1991; Redgwell et al., 1997). The significance and cause of the galactosyl reduction throughout fruit development and the increased rate of reduction during ripening has not been established. However, cell wall galactosyl residue turnover occurs throughout fruit development, and it seems likely that an increase in the rate of degradation is responsible for the increased rate of removal during ripening. In ripening fruit, physiological studies have shown a 4- to 5-fold increase in exo-galactanase activity, coupled with a corresponding increase in free Gal (Pressey, 1983; Kim et al., 1991; Carey et al., 1995).

At least seven tomato β-galactosidase (TBG) genes are expressed during fruit development (Smith and Gross, 2000). Six are known to be expressed during ripening, and of these six, the products of five are predicted to be localized in the wall. The expression

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of one of these genes was recently suppressed by producing plants containing a partial sense TBG1 transgene (Carey et al., 2001). However, even when TBG1 mRNA was suppressed to 10% of control levels, no changes in texture or cell wall composition were detected in transgenic fruit. The function of the TBG3 gene product has also been investigated by antisense suppression. Transgenic fruit with reduced TBG3 mRNA had reduced exo-galactanase activity and an increase in wall galactosyl content. Although antisense fruit were not significantly firmer than controls, they deteriorated more slowly during long-term storage and processed into pastes with an increased proportion of insoluble solids and slightly increased viscosity (de Silva and Verhoeyen, 1998).

When fruit development was staged chronologically (dpp) in mutant and wild-type fruit, TBG4 was the only ripening-related β-galactosidase gene with attenuated transcript abundance in fruit of the ripening mutants *rin* and *nor* (Smith and Gross, 2000). Furthermore, the exo-galactanase activity of β-galactosidase II (TBG4-encoded gene product), was shown to increase more than 4-fold during ripening in wild type but not *rin* and *nor* fruit (Carey et al., 1995). To assess the contribution of the TBG4-encoded product to cell wall properties and fruit firmness, antisense experiments were carried out to suppress TBG4 mRNA accumulation during fruit ripening.

RESULTS

Suppression of TBG4 Transcript Accumulation

To suppress the accumulation of TBG4 mRNA in tomato fruit, a binary vector was constructed using the cauliflower mosaic virus (CaMV) 35S promoter to express a 1.5-kb segment of the cDNA in the antisense orientation. *Agrobacterium tumefaciens*-mediated transformation and tissue culture regeneration were used to introduce T-DNA harboring a kanamycin resistance gene and the antisense construct into 20 independent lines (T_0 lines). T_0 plants were grown to maturity, and T_1 seed was collected and plated on kanamycin-containing medium. Only lines having a 3:1 kanamycin resistant:sensitive ratio, indicative of a single-locus insertion of the T-DNA, were subjected to further analysis. Southern analysis was performed to determine the T-DNA copy number and integrity (data not shown). A total of six single-insertion site lines were chosen for further analysis. Control lines consisted of progeny from a self of the parental line used for transformations and an azygous line derived by rescuing two T_1 kanamycin-sensitive plants each from three antisense lines.

Fruit were harvested from control and antisense plants at mature green stage 4 (MG4; approximately 42 dpp), breaker plus 3 d (B3), and breaker plus 7 d (B7), and mRNA levels were determined by RNA gel-blot analysis (Fig. 1). To reduce potential cross-hybridization to other TBG genes, the 3′-untranslated region of the TBG4 cDNA was used as a template to synthesize the probe. TBG4 mRNA levels were low in all the lines in green fruit at MG4, confirming previous observations (Smith and Gross, 2000). At the B3 stage, TBG4 transcript abundance was reduced in all antisense lines (Fig. 1). The greatest suppression was observed in line 1-1, where TBG4 mRNA abundance was reduced to 15% of control fruit. The suppression of TBG4 mRNA abundance was unexpectedly not observed in B7 stage fruit of line 1-1, and in four antisense lines (1-1, 2-11, 4-2, and 4-5), TBG4 mRNA levels were higher than those observed in control fruit. RNA gel-blot analysis, as described for Figure 1, was also performed using the full-length TBG4 cDNA as a probe. The full-length probe detected both the endogenous 2.8-kb TBG4 mRNA at levels approximately equal to those shown in Figure 1 and 1.5-kb antisense transcript in all the transgenic lines, except line 1-1, where no antisense transcript, at any stage was ever detected (data not shown).

RNA gel-blot analysis was also carried out to assess the effect of TBG4 antisense on the abundance of other TBG mRNAs, because the TBG4 cDNA shares approximately 70% nucleotide sequence identity to other β-galactosidase gene family members (Smith and Gross, 2000). Note that the method used for RNA gel-blot analysis shown in Figure 2 utilized a more

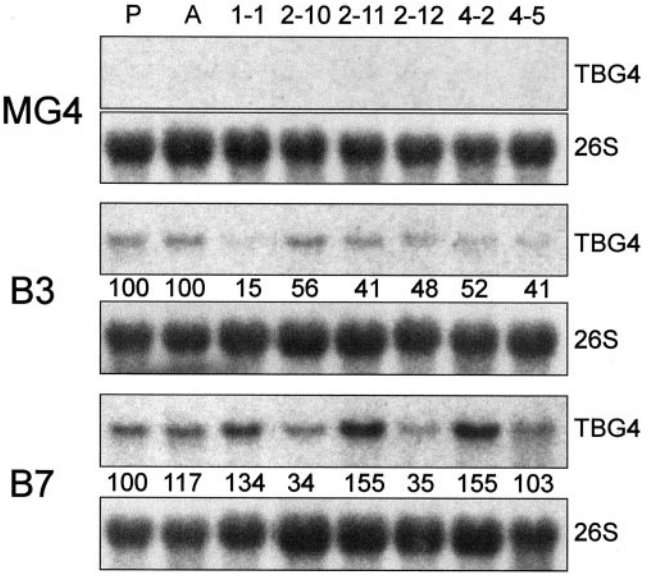

Figure 1. RNA gel-blot analysis of TBG4 mRNA levels in control and antisense fruit. Twenty micrograms of total RNA extracted from fruit at stages indicated on the left was loaded in each lane. Lines include the parental (P), azygous (A), and transgenic lines indicated on top. Blots were hybridized using the probes indicated on the right. Scanning densitometry was done for the B3 and B7 samples to quantify relative TBG4 mRNA levels. The parental (P) line TBG4 mRNA level was arbitrarily set at 100, and the numbers under each lane are the percent band density when compared with the parental line. A 26S ribosomal gene clone from soybean (*Glycine max*) was used as a loading control for scanning densitometry.

sensitive protocol than that shown in Figure 1 (see "Materials and Methods"). The increased sensitivity allowed quantification of mRNA abundance at the MG4 stage. The mRNA abundance of TBG1, TBG2, TBG3, TBG4, TBG5, and TBG6 was determined using RNA from the parental and the most highly suppressed antisense line 1-1 (Fig. 2). The mRNA abundance of TBG2, TBG5 and TBG6 was essentially the same in line 1-1 versus control fruit (Fig. 2). However, TBG3 mRNA abundance was significantly attenuated in line 1-1 fruit but only at MG4. Interestingly, TBG3 mRNA levels in line 1-1 were significantly higher than in control fruit at B3 and B7 and TBG1 mRNA abundance was 2-fold higher in line 1-1 fruit, but only at B7 (Fig. 2). As expected, TBG4 mRNA abundance was reduced in line 1-1 fruit to 15% of that found in control fruit at MG4 (Fig. 2.). In contrast to the data shown in Figure 1, TBG4 mRNA abundance was not attenuated in line 1-1 when compared with control fruit at B3 (Fig. 2). Note that the fruit harvested for the analysis shown in Figure 1 were collected in the winter, whereas fruit collected for Figure 2 were harvested in the spring from a second planting of T_1 and control plants.

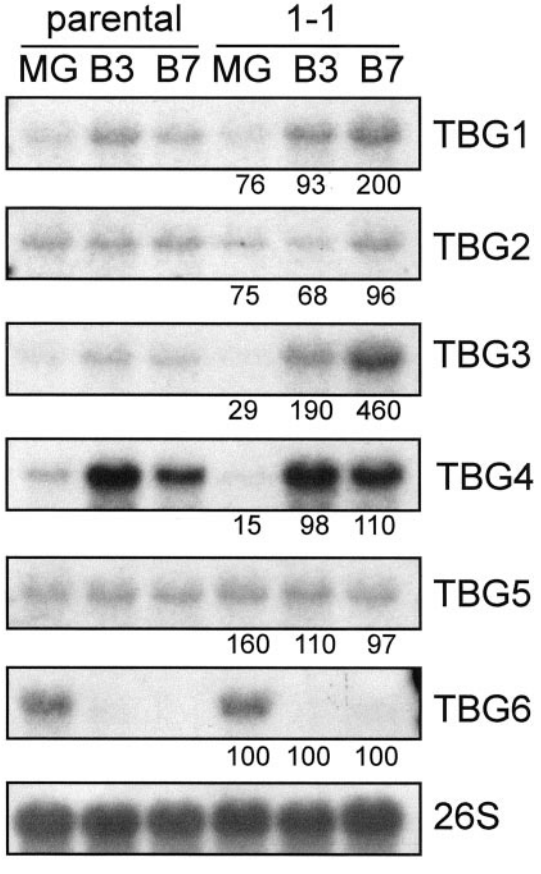

Figure 2. RNA gel-blot analysis of other TBG mRNA levels in control and antisense line 1-1 fruit. Twenty micrograms of total RNA extracted from fruit at stages indicated on the top was loaded in each lane. Blots were hybridized using the probes indicated to the right. Scanning densitometry was done for samples to quantify relative TBG mRNA levels. The numbers under each lane for line 1-1 represent the percent band density when compared with the control parental line at the equivalent stage. A 26S ribosomal gene clone from soybean was used as a loading control for scanning densitometry, and one example is shown.

Suppression of TBG4 mRNA Reduced Exo-Galactanase Activity, But Not Total β-Galactosidase Activity

To clarify the work presented here, the following terms are used: β-galactosidase, an enzyme that can hydrolyze a β-galactosyl residue linked to a variety of aglycones (e.g. lactose, *p*-nitrophenyl-β-D-galactoside [PNP-gal], etc.); exo-galactanase, an enzyme that is specific for the non-reducing end of galactan, has no action on PNP-gal or lactose, and whose affinity for the substrate increases the depolymerization of the substrate; and galactanase, an enzyme that cleaves internal bonds in galactan. Moreover, an enzyme that can hydrolyze galactose from PNP-gal and the non-reducing end of galactan is referred to as a β-galactosidase/exo-galactanase (B. Henrissat, personal communication; Henrissat, 1998).

Protein extracts were prepared from fruit at the B3 and B7 stages of ripening and assayed for β-galactosidase and exo-galactanase activity using PNP-gal and lupin galactan as substrates, respectively (Fig. 3, A and B). Although TBG4 mRNA levels were reduced in fruit of all antisense lines at the B3 stage, total β-galactosidase activity was similar, with one exception, in all lines at both the B3 and B7 stage (Fig. 3A). The single exception was line 1-1, where B7 fruit had nearly twice as much total β-galactosidase activity than at the B3 stage (Fig. 2).

A correlation between reduced TBG4 mRNA levels and reduced exo-galactanase activity was observed in B3 stage fruit (Fig. 3B). For example, among the antisense lines, line 1-1 which had the lowest mRNA levels (Fig. 1), also had the lowest exo-galactanase activity. The remaining antisense lines, which had approximately 40% to 50% of normal TBG4 mRNA levels, also had greater than 50% reduction in exo-galactanase activity in B3 stage fruit. Although the lines with the lowest TBG4 mRNA accumulation also had the lowest exo-galactanase activity, the suppression of exo-galactanase activity was greater than the suppression of TBG4 mRNA accumulation relative to controls. However, all the antisense lines, except line 2-12, had exo-galactanase activity levels comparable with control fruit at the B7 stage. This observation is consistent with the fact that all of the antisense lines, except 2-10 and 2-12, had normal TBG4 mRNA levels at the B7 stage. Interestingly, although line 2-10 and 2-12 both had reduced TBG4 mRNA levels in B7 stage fruit, only line 2-12 had a corresponding reduction in exo-galactanase activity.

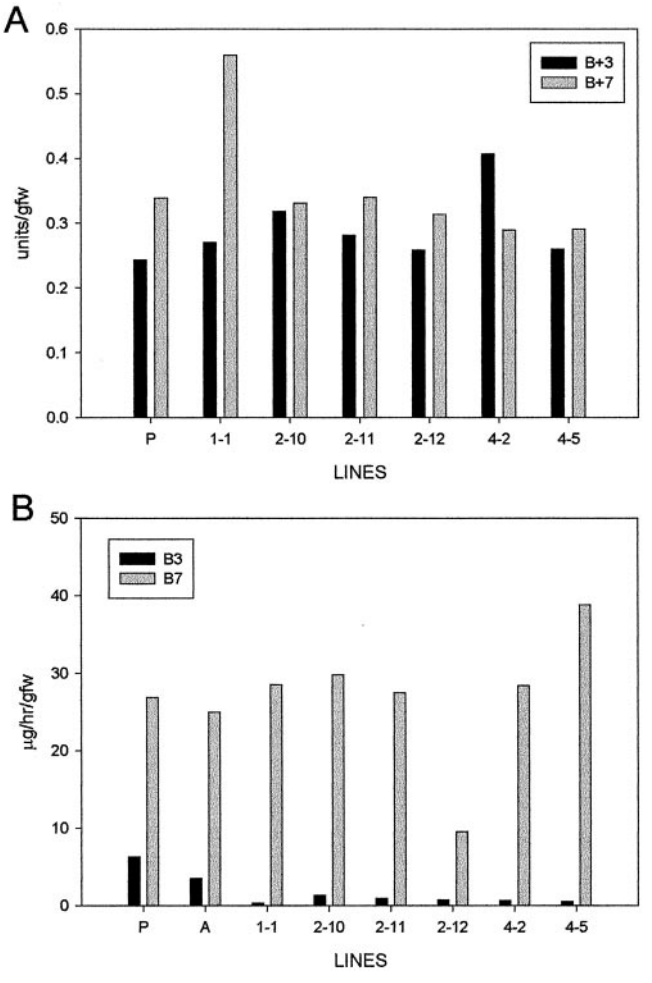

Figure 3. Control and antisense fruit β-galactosidase and exo-galactanase activity. Protein extracts were made from control and antisense fruit tissues at the stages indicated and assayed for total β-galactosidase activity against a PNP-gal substrate (A) and exo-galactanase activity against a lupin galactan (B). Linear time points for data analysis were empirically determined for both assays and β-galactosidase and exo-galactanase assays were terminated after 1 and 4 h at 37°C, respectively.

Lower Exo-Galactanase Activity Results in Transient Reduction in Free Gal, But Only Minor Changes in Total Cell Wall Galactosyl Content

The level of free Gal (product of exo-galactanase activity) rises throughout ripening in normal tomato fruit. A reduction in cell wall-localized exo-galactanase activity might be expected to result in reduced levels of free Gal in ripening fruit. MG4 stage fruit from all of the antisense lines had approximately 50% less free Gal when compared with control lines (Fig. 4). However, no significant reduction in free Gal was observed in B3 or B7 stage fruit in any of the antisense lines. A subtle, but possibly significant, observation noted among some of the antisense lines was that free-Gal levels did not increase from the B3 to B7 stage in lines 1-1, 2-10, and 2-12.

Increased free-Gal levels were shown to hasten the ripening and affect C_2H_4 production of tomato fruit (Gross, 1985). Therefore, fruit from each line were harvested at the breaker stage and monitored for ethylene and CO_2 production for 10 d. Two fruit from each line were monitored on two separate occasions. No significant differences were observed in either the time to reach peak or level of peak C_2H_4 and CO_2 production between control and antisense fruit (data not shown).

Although total free-Gal levels were lower in fruit of the antisense lines at MG4, the total cell wall galactosyl content (as percentage of wall weight) in antisense fruit was comparable with controls (Fig. 5). However, during ripening, no reduction in galactosyl content of line 1-1 occurred as it did in controls and other antisense lines. Moreover, at the B7 stage, line 1-1 had nearly twice the cell wall galactosyl content compared with the other lines.

Whole-Fruit Firmness

The mean peak force required to compress whole fruit 3 mm at an equatorial position centered over a locule was determined using two different probes (Fig. 6, A and B). Two measurements were made on each fruit, with each probe, and at least 15 fruit from five plants were tested from each line. Data from both the A, flat plate (comparable with a whole-hand squeeze), and B, spherical indenter (comparable with a finger press), probes detected similar differences in firmness in fruit among the lines. All of the antisense lines had firmer fruit than control fruit. However, the mean peak force of lines 4-2 and 4-5 were not significantly different from control fruit. The firmest line,

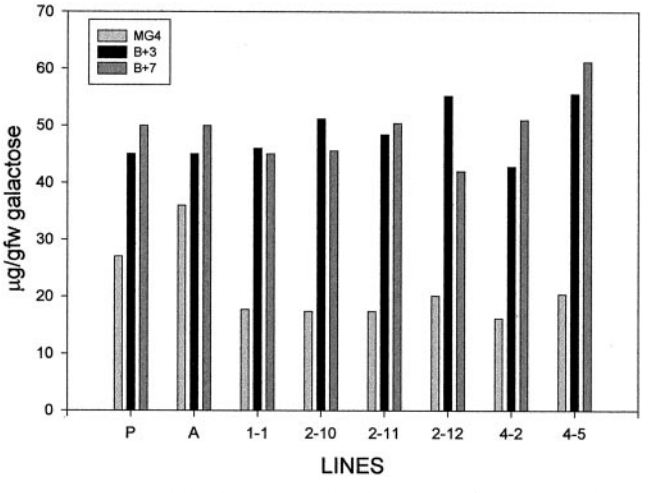

Figure 4. Free Gal levels in control and antisense fruit. Free Gal was identified and quantified by gas chromatography/mass spectrometry-selected ion monitoring of the alditol acetate derivatives from a sample of the 40% (v/v) ethanol-soluble extracts of peel and outer pericarp tissues.

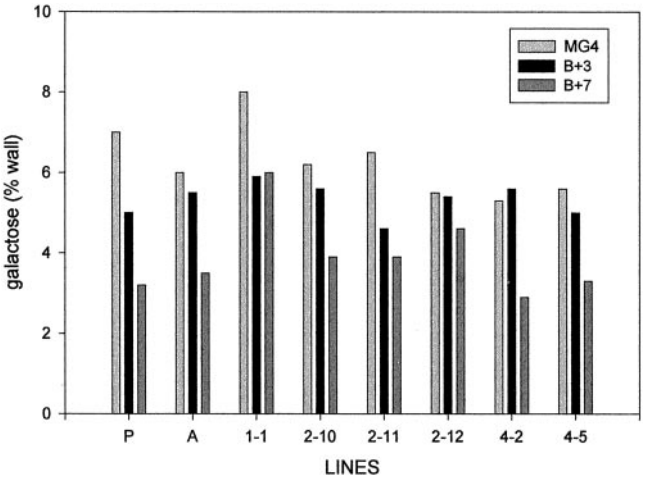

Figure 5. Cell wall galactosyl content in control and antisense fruit. Galactosyl content is presented as percent Gal, by weight, of the total wall.

1-1, had a mean peak force using the flat plate probe that was 40% higher than azygous control fruit. Antisense lines 2-10, 2-11, and 2-12 had mean peak force measurements that were 27%, 26%, and 33% firmer than azygous fruit.

The control and antisense fruit used for texture measurements were also examined for other ripening characteristics such as the number of days from pollination to breaker, weight, and color at the B7 stage (Table I). No prominent differences in these ripening characteristics were observed between the control or antisense lines, although some minor variations were observed. Antisense line 4-2 had fruit with an average weight less than the other lines. However, fruit from line 4-2 had a normal shape, number of seeds, ripening rate and color when compared with the other lines. Furthermore, the average number of days to reach the breaker stage was similar when comparing the parental line to the antisense lines, whereas fruit from the azygous line reached the breaker stage more quickly. Because of their delayed growth when rescued from kanamycin-containing plates, plants of the azygous line were grown approximately 2 months after the other lines. Fruit from the parental and antisense lines were harvested in late December and January, whereas the azygous line fruit were harvested in March, at a time with more intense and longer period of natural daylight.

DISCUSSION

TBG4 codes for β-galactosidase II, an enzyme known to have β-galactosidase and exo-galactanase activity, and likely to be prominent in wall galactosyl turnover during ripening (Carey et al., 1995; Carrington and Pressey, 1996; Smith et al., 1998; Smith and Gross, 2000). To help determine the function of the TBG4 gene product during fruit ripening, we created transgenic tomato plants expressing a segment of the cDNA in the antisense orientation.

All of the antisense lines had fruit with general ripening characteristics comparable with control fruit (Table I). All of the antisense lines examined had reduced levels of TBG4 mRNA in fruit at the B3 stage (Fig. 1), but only two lines (2-10 and 2-12) had reduced levels of mRNA at B7. As expected, all of the antisense lines had reduced levels of exo-galactanase activity, except line 2-4, at the B3 stage and normal levels at B7. The reduction in exo-galactanase activity at the B3 stage was comparable with or greater than the reduction in TBG4 mRNA. Total β-galactosidase activity was essentially unchanged in antisense fruit at both the B3 and B7 stage. The single exception was observed for line 1-1 where B7 fruit had nearly twice as much total β-galactosidase activity as fruit at the B3 stage (Fig. 3A), and this may be correlated to the significant increase of TBG3 mRNA abundance

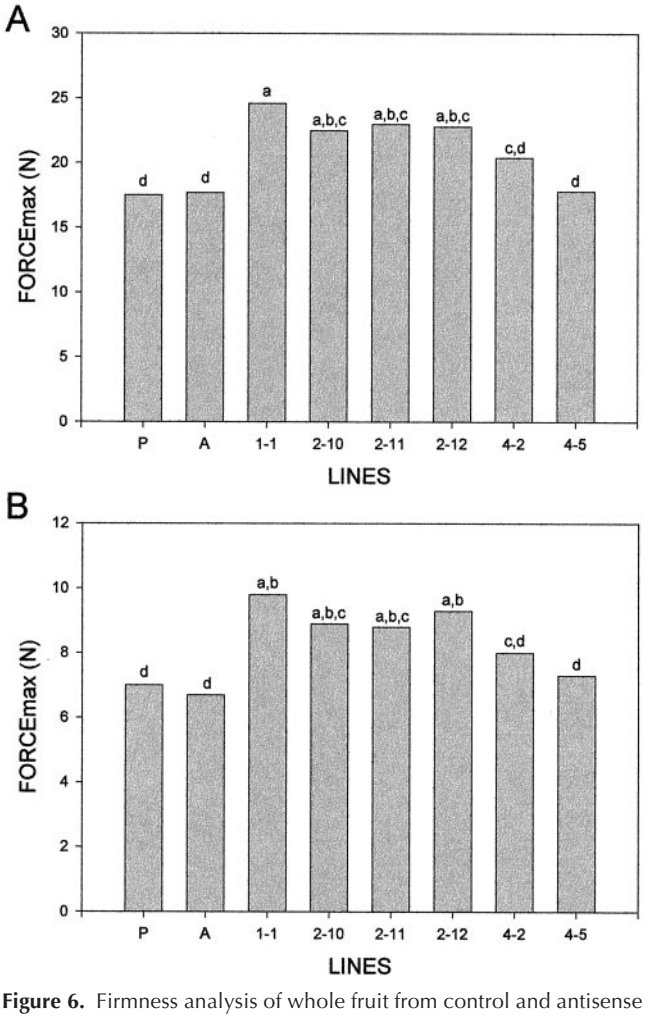

Figure 6. Firmness analysis of whole fruit from control and antisense lines. Two measurements were made using each probe on each of at least 15 fruit per line. Means with the same letter are not significantly different; $P < 0.05$, Sheffé test. A, Mean of maximum force from flat plate compression tests. B, Mean of maximum force from spherical indenter compression tests.

Table I. *Fruit ripening characteristics*
ND, Not determined.

Line	DTB	Wt	HunterA
		g	
Parental	52	175	ND
Azygous	44	142	32.2
1-1	50	175	30.2
2-10	51	182	29.6
2-11	52	188	28.8
2-12	49	162	30.4
4-2	51	108	30.7
4-5	49	142	32.2

observed in line 1-1 at B7 (Fig. 2). We have recently expressed the TBG3 gene product in yeast and have determined that although the enzyme has β-galactosidase activity, no exo-galactanase activity against a lupine galactan or native tomato fruit pectin fractions was detected (data not shown). Furthermore, in fruit from the ripening mutants *rin* and *nor* at a time chronologically equivalent to ripening wild-type fruit, only exo-galactanase and not β-galactosidase activity is reduced, and TBG4 is the only β-galactosidase gene family member with a significantly attenuated mRNA level (Carey et al., 1995; Smith and Gross, 2000). Taken together, these observations suggest that the TBG4-encoded product, although responsible for the majority of detectable exo-galactanase activity, does not contribute significantly to the total detectable β-galactosidase activity during fruit ripening.

Free-Gal levels in TBG4 antisense fruit just before ripening were reduced approximately 50% compared with control fruit. No significant differences in free-Gal levels were detected at the B3 or B7 stage. The reduction in TBG4 mRNA and corresponding reduction in exo-galactanase among the antisense lines at B3 unexpectedly did not result in reduced free Gal, although a rise in free Gal between B3 and B7 was not seen in the most strongly suppressed lines. The source and mechanism of free-Gal increase during tomato fruit ripening is unknown, but it has been hypothesized that the rise in exo-galactanase activity may result in a decrease in wall galactosyl content during ripening, leading to the rise in free Gal (Gross, 1983). It is possible that reduced levels, or the delayed appearance of detectable levels of TBG4 mRNA at MG4 among the antisense lines resulted in the transient reduction in free-Gal levels. However, this does not explain why free-Gal levels are comparable with control fruit at the B3 stage, at a time when TBG4 mRNA and exo-galactanase levels were at their lowest among the antisense lines. The TBG4 encoded protein, when produced using a yeast expression system, is very stable and high levels of activity are maintained even after 2 weeks at room temperature (data not shown). This observation suggests that the TBG4-encoded enzyme may be very stable in tomato fruit, and even when mRNA levels are low in antisense fruit, enzyme levels and activity may continue to accumulate.

During ripening total cell wall galactosyl content of the antisense lines (except 1-1) was similar to control fruit. In the most strongly suppressed lines (1-1, 2-11, and 2-12), free-Gal levels did not increase between B3 and B7, as it did in the controls and remaining antisense lines. Although line 1-1 did have the firmest fruit, other antisense lines also had fruit significantly firmer than controls, but did not differ significantly in total wall galactosyl content. Therefore, no significant consensus among the antisense lines between increased wall galactosyl content and fruit firmness was observed. We are currently examining the galactosyl content of different cell wall fractions before and after ripening to determine whether an increase in galactosyl content of a specific cell wall fraction among the antisense lines can be correlated with increased fruit firmness.

Fruit from all of the TBG4 antisense lines were firmer than control fruit. Among the antisense lines with substantially firmer fruit, line 1-1 had a mean peak force 40% higher than control fruit. Correspondingly, line 1-1 had the lowest TBG4 mRNA and exo-galactanase activity levels at the B3 stage and also had a higher wall galactosyl content at MG4 and B7. However, in none of the antisense lines was TBG4 mRNA suppressed during all the stages of ripening. Two of the six antisense lines that had suppressed TBG4 mRNA in B3 fruit, did not have significantly firmer fruit. Thus, lines 4-2 and 4-5 might have had low TBG4 mRNA levels during ripening but not before. We are currently examining the regulation of TBG4 expression. Using more sensitive detection methods, we found that TBG4 mRNA levels are low but clearly detectable in MG4 (Fig. 2) and immature green fruit (data not shown). Taken together with the data from the antisense lines, this observation suggests that any correlation between increased fruit firmness and TBG4 mRNA suppression likely results from TBG4 mRNA suppression before or at the onset of ripening. The differences observed in the TBG4 mRNA abundance in line 1-1 fruit at B3 (compare Figs. 1 and 2) could be attributable to slight variability in staging the fruit collected for the two experiments. As an alternative, the fruit used for RNA extractions in Figure 1 were harvested in the winter, whereas fruit for Figure 2 were harvested in the spring. It is possible and likely that fruit ripen a bit faster in the spring than winter and that B3 in spring may be equal to B4 in winter. Nevertheless, this difference highlights the instability of suppressing TBG4 mRNA levels in the antisense lines.

It is known that at least six additional β-galactosidase genes are expressed in fruit and that the TBG4 antisense DNA has up to 70% shared sequence identity with the other gene family members. Therefore, the dramatic increase in firmness of antisense line 1-1, but lack of dramatic enzyme activity or biochem-

ical composition could be attributable to alteration in a combination of β-galactosidase gene family member mRNA levels. Other than TBG4, only TBG3 mRNA abundance was found to be significantly attenuated in antisense line 1-1 at MG4 (Fig. 2). It is possible that the combination of TBG4 and TBG3 mRNA attenuation before or at the onset of ripening is required for increased fruit firmness. However, this is unlikely because the native substrate specificity of the TBG3 gene product is not known, and as noted above, we did not detect exo-galactanase activity from the TBG3 product expressed in yeast. Furthermore, in transgenic antisense experiments of the TBG3 gene in tomato, it was found that in addition to suppression of TBG3 mRNA levels, both TBG1 and TBG4 mRNAs were suppressed (de Silva et al., 1998). Interestingly, even though the TBG1 gene product was shown to have exo-galactanase activity, in transgenic lines where TBG1 was suppressed to 10% of wild-type levels, no change in exo-galactanase activity or changes in cell wall galactosyl content were observed (Carey et al., 2001). Thus, the TBG4 gene product is clearly implicated in providing the vast majority of detectable exo-galactanase activity and cell wall-related galactosyl changes throughout ripening.

Presence of the TBG4 antisense construct is linked to significantly firmer fruit in four of the six antisense lines presented here. However, there are no clear correlations linking the biochemical data to the increased firmness among all the antisense lines when compared with controls. The best suggestion that the TBG4 gene product directly affects fruit firmness is that the firmest antisense line, 1-1, also had the lowest TBG4 mRNA and exo-galactanase activity and highest cell wall galactosyl content before and during the early stages of ripening. The reason(s) why reduced exo-galactanase activity might result in increased fruit firmness remains speculative. Exo-galactanase activity is believed to be responsible for reducing wall galactosyl levels in a variety of fruits including tomato (Gross and Sams, 1984; Redgwell et al., 1997). An increased content of pectic galactan side chains has been shown to be linked with increased mechanical strength of the wall in pea cotyledons (McCartney et al., 2000). Therefore, any correlation with reduced levels of exo-galactanase activity during the early stages of ripening and fruit firmness during the later stages of ripeness might suggest that galactosyl-containing side chains in the wall result in decreasing wall porosity, thereby obstructing access to wall components by other wall hydrolases and preventing depolymerization of structural polysaccharides (Redgwell et al., 1997; Carpita and McCann, 2000; Brummell and Harpster, 2001). A detailed examination of the composition and size of various cell wall fractions among the control and antisense lines will be necessary to determine whether any correlation can be made between reduced TBG4 encoded exo-galactanase activity, cell wall composition, and fruit firmness.

MATERIALS AND METHODS

Plant Material and Transformations

Tomato (*Lycopersicon esculentum* Mill. cv Rutgers) seed was acquired from Meyer Seed Co. (Baltimore). Plants were grown in a greenhouse using standard cultural practices. Flowers were tagged after hand pollination, and fruit were harvested according to the number of dpp or days post-breaker stage.

The binary vector pBI121 (CLONTECH, Palo Alto, CA) was modified by removing the β-glucuronidase coding region by digestion with *Bam*HI and *Sac*I and inserting a 1,524-bp fragment from the 5′ end of the TBG4 cDNA (accession no. AF020390) in the antisense orientation with respect to the 35S CaMV promoter. Expression and termination were provided by the 35S CaMV promoter and nopaline synthase terminator present in pBI121. *Agrobacterium tumefaciens*-mediated transformation and regeneration of plantlets was done using strain LBA4404 and cotyledons from 7-d-old seedlings using the method of Deikman and Fischer (1988), except that tobacco (*Nicotiana tabacum*) feeder cells were not used. Transformation was confirmed by performing DNA gel-blot analysis using genomic DNA isolated from leaves of T_0 plants. Only plants containing single T-DNA insertion sites were used for further evaluation.

Five or six T_1 generation plants were grown for each line. Seed from each T_1 plant was collected and plated on kanamycin-containing medium to determine whether it was heterozygous or homozygous for the T-DNA insertion. Three to five fruit were collected from each plant for textural analysis, and at least five fruit from at least three plants of each line were collected for biochemical analysis. Control plants consisted of (a) parental line, the pooled progeny from the self of five plants grown from the purchased seed, and (b) azygous, kanamycin-sensitive T_1 plants of lines 2-10, 2-11, and 2-12 (described below) rescued from selective media.

RNA Extraction and Gel-Blot Analysis

Fruit were harvested at 42 dpp, B3, and B7, chilled on ice, processed to include only outer pericarp and peel tissues, frozen in liquid nitrogen, and stored at −80°C. RNA was extracted and RNA gel-blot analysis was carried out using methods previously described (Smith and Gross, 2000). In brief, blots were prepared from gels containing 20 μg of total RNA per lane. After transfer, the membranes were washed for 1 min in 0.3 M Na-acetate (pH 5.2), and briefly stained in 0.02% (w/v) methylene blue/0.3 M Na-acetate (pH 5.2) solution to evaluate the transfer and visualize and mark the 18S and 27S ribosomal RNAs and a standard RNA ladder. The membranes were then washed several times with 0.2× SSC with 0.5% (w/v) SDS to remove the methylene blue stain. Probes were synthesized using a random primed DNA labeling kit (Roche Molecular Biochemicals, Indianapolis), the 3′-untranslated region of the TBG cDNAs as a template, and [α-^{32}P]dATP (3,000 Ci mmol^{-1}). All blots were washed to a final stringency using 0.1× SSC with 0.2% (w/v) SDS at 65°C twice for 20 min each. Biomax MS x-ray film (Eastman-Kodak, Rochester, NY) was used and exposed 36 h for TBG1, TBG4, and TBG6 and 72 h for TBG2, TBG3, and TBG5 at −80°C using an intensifying screen. As a loading control, RNA blots were stripped and reprobed at a reduced hybridization and washing stringency using a soybean (*Glycine max*) 26S rDNA fragment. Autoradiographic data were quantified using a Fluor-S MultiImager and Quantity One software (Bio-Rad, Hercules, CA).

To analyze the accumulation of other TBG mRNAs (Fig. 2), a more sensitive RNA gel-blot method was used. This method was essentially the same as above, except that total RNA was fixed to the nylon membrane using an UV cross-linker (GS Gene Linker UV Chamber, Bio-Rad) at 70 mJ for 4 min, before methylene blue staining. The remaining procedure was the same as described above.

Enzyme Assays, Cell Wall Preparations, and Free-Gal Determination

Enzyme extractions were made from homogenized peel and pericarp tissues following the methods described by Carey et al. (1995). β-Galactosidase activity was determined as described by Pressey (1983) using PNP-gal as

substrate; 1 unit of activity was defined as the amount of enzyme that liberated 1 μmol PNP min^{-1} at 37°C. Exo-galactanase activity was measured against a lupin galactan pretreated with α-L-arabinofuranosidase (Megazyme, Wicklow, Ireland) following previously described methods (Carey et al., 1995). Pilot assays were done to confirm that enzyme activities were determined at a time point when activity was increasing linearly.

Cell wall purification and free-Gal preparations were made using peel and outer pericarp tissues following the protocols in Gross (1983, 1984). The sugar composition of the acetone insoluble wall material was determined after H_2SO_4 hydrolysis. Free monosaccharides were identified and quantified by gas chromatography/mass spectrometry-selected ion monitoring of the alditol acetate derivatives (Gross and Acosta, 1991).

Texture, Color, and Respiration Measurements

Firmness and force relaxation characteristics were measured on 15 to 20 intact tomatoes (at least two from each of five plants) from each line, harvested at the B7 stage. Each fruit was tested twice by flat plate compression and twice by indentation with a 6.35-mm spherical probe centered over different locules. We felt that the small deformations used would have minimal lateral effect and would not affect adjacent indentation measurements.

Force-deformation curves were recorded using a universal testing (force/deformation) instrument (TA-XT2i, Stable Microsystems Texture Analyzer, Surrey, UK) loading at 1 mm s^{-1} to 3 mm and then after stress relaxation for 10 s. Firmness was defined as force at maximum deformation (3 mm) and is an elastic property; whereas relaxation parameters represent viscoelastic properties. We analyzed the relaxation portions of the curves using an empirical 3-parameter equation developed by Wu and Abbott (2002). Firmness and relaxation parameters were not significantly correlated, and so represent relatively unrelated mechanical properties. The relaxation parameters did not differ significantly among lines, in general agreement with the findings of Errington et al. (1997), Langley et al. (1994), and Tieman and Handa (1994) on polygalacturonase- and pectin methylesterase-antisense transformed tomatoes; therefore, measurement of relaxation was discontinued. Peak force measurements were used for statistical analysis using SAS software (SAS Institute, Cary, NC).

Color development was monitored using a Minolta colorimeter by taking three measurements on the blossom end of each fruit used for texture analysis.

Ethylene and carbon dioxide production were monitored using an automatic sampling, flow-through system similar to that described previously (Watada and Massie, 1981). Two fruit were monitored separately from each line in two separate experiments. The data presented were typical for all fruit tested.

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LITERATURE CITED

Brummell DA, Harpster MH (2001) Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. Plant Mol Biol **47:** 311–340

Brummell DA, Harpster MH, Civello PM, Palys JM, Bennett AB, Dunsmuir P (1999) Modification of expansin protein abundance in tomato fruit alters softening and cell wall polymer metabolism during ripening. Plant Cell **11:** 2203–2216

Carey AT, Holt K, Picard S, Wilde R, Tucker GA, Bird CR, Schuch W, Seymour GB (1995) Tomato exo-β(1→4)-D-galactanase: isolation, changes during ripening in normal and mutant tomato fruit, and characterization of a related cDNA clone. Plant Physiol **108:** 1099–1107

Carey AT, Smith DL, Harrison E, Bird CR, Gross KC, Seymour GB, Tucker GA (2001) Down-regulation of a ripening-related β-galactosidase gene (TBG1) in transgenic tomato fruits. J Exp Bot **52:** 663–669

Carpita N, McCann M (2000) The cell wall. *In* BB Buchanan, W Gruissem, RL Jones, eds, Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologists, Rockville, MD, pp 52–108

Carrington CMS, Pressey R (1996) β-Galactosidase II activity in relation to changes in cell wall galactosyl composition during tomato ripening. J Am Hortic Soc **121:** 132–136

Deikman J, Fischer RL (1988) Interaction of a DNA binding factor with the 5′-flanking region of an ethylene-responsive fruit ripening gene from tomato. EMBO J **7:** 3315–3320

de Silva J, Verhoeyen ME (1998) Production and characterization of antisense-exogalactanase tomatoes. *In* HA Kuiper, ed, Report of the Demonstration Programme on Food Safety Evaluation of Genetically Modified Foods as a Basis for Market Introduction. The Netherlands Ministry of Economic Affairs, The Hague, pp 99–106

Errington N, Mitchell JR, Tucker GA (1997) Changes in the force relaxation and compression responses of tomatoes during ripening: the effect of continual testing and polygalacturonase activity. Postharvest Biol Technol **11:** 141–147

Gross KC (1983) Changes in free galactose, myo-inositol and other monosaccharides in normal and non-ripening mutant tomatoes. Phytochemistry **22:** 1137–1139

Gross KC (1984) Fractionation and partial characterization of cell walls from normal and non-ripening mutant tomato fruit. Physiol Plant **62:** 25–32

Gross KC (1985) Promotion of ethylene evolution and ripening of tomato fruit by galactose. Plant Physiol **79:** 306–307

Gross K, Acosta PB (1991) Fruits and vegetables are a source of galactose: implications in planning the diets of patients with galactosemia. J Inherited Metab Dis **14:** 253–258

Gross KC, Sams CE (1984) Changes in cell wall neutral sugar composition during fruit ripening: a species survey. Phytochemistry **23:** 2457–2461

Henrissat B (1998) Glycosidase families. Biochem Soc Trans **26:** 153–156

Kim J, Gross KC, Solomos T (1991) Galactose metabolism and ethylene production during development and ripening of tomato fruit. Postharvest Biol Technol **1:** 67–80

Kramer M, Sanders R, Bolkan H, Waters C, Sheehy RE, Hiatt WR (1992) Postharvest evaluation of transgenic tomatoes with reduced levels of polygalacturonase: processing, firmness and disease resistance. Postharvest Biol Technol **1:** 241–255

Langley KR, Martin A, Stenning R, Murray AJ, Hobson GE, Schuch WW, Bird CR (1994) Mechanical and optical assessment of the ripening of tomato fruit with reduced polygalacturonase activity. J Sci Food Agric **66:** 547–554

McCartney L, Ormerod AP, Gidley MJ, Knox JP (2000) Temporal and spatial regulation of pectic (1–4)-β-D-galactan in cell walls of developing pea cotyledons: implications for mechanical properties. Plant J **22:** 105–113

Pressey R (1983) β-Galactosidases in ripening tomatoes. Plant Physiol **71:** 132–135

Redgwell RJ, Fischer M, Kendal E, MacRae EA (1997) Galactose loss and fruit ripening: high-molecular-weight arabinogalactans in the pectic polysaccharides of fruit cell walls. Planta **203:** 174–181

Schuch W, Kanczler J, Robertson D, Hobson G, Tucker G, Grierson D, Bright S, Bird C (1991) Fruit quality characteristics of transgenic tomato fruit with altered polygalacturonase activity. HortScience **26:** 1517–1520

Smith DL, Gross KC (2000) A family of at least seven β-galactosidase genes is expressed during tomato fruit development. Plant Physiol **123:** 1173–1183

Smith DL, Starrett DA, Gross KC (1998) A gene coding for tomato fruit β-galactosidase II is expressed during fruit ripening. Plant Physiol **117:** 417–423

Thakur BR, Singh RK, Handa AK (1996) Effect of an antisense pectin methylesterase gene on the chemistry of pectin in tomato (*Lycopersicon esculentum*) juice. J Agric Food Chem **44:** 628–630

Tieman DM, Handa AK (1994) Reduction in pectin methylesterase activity modifies tissue integrity and cation levels in ripening tomato (*Lycopersicon esculentum* Mill.) fruits. Plant Physiol **106:** 429–436

Tieman DM, Harriman RW, Ramamohan G, Handa AK (1992) An antisense pectin methylesterase gene alters pectin chemistry and soluble solids in tomato fruit. Plant Cell **4:** 667–679

Watada AE, Massie DR (1981) A compact automatic system for measuring CO_2 and C_2H_4 evolution by harvested horticultural crops. HortScience **16:** 39–41

Wu T, Abbott JA (2002) Firmness and force relaxation characteristics of tomatoes stored intact or as slices. Postharvest Biol Technol **24:** 59–68